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Partial Digestion of tRNA-Aminoacyl-tRNA Synthetase Complexes with Cobra Venom Ribonuclease†

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ABSTRACT: Transfer RNA molecules have been labeled with ^{32}P at the 5′ or 3′ end and digested with cobra venom ribonuclease, which preferentially cuts double-stranded regions. The products of yeast $tRNA^{Phe}$ and $tRNA^{Val}$ were analyzed by high-resolution gel electrophoresis. In the free state, these tRNAs were cut predominantly in the acceptor and anticodon stems. Minor cuts occurred in the TΨ stem in $tRNA^{Val}$. The topography of zones interacting with their cognate synthetases was studied by determining the tRNA regions shielded by protein. Nearly 100% protection was found in the anticodon and acceptor stem of $tRNA^{Val}$, while in $tRNA^{Phe}$ only the stem of the anticodon was protected. Noncognate interactions between $tRNA^{Phe}$ and tryptophanyl-tRNA synthetase from beef pancreas were examined. The beef enzyme did not protect $tRNA^{Phe}$ despite the fact that efficient misaminoacylation occurred. The pattern of shielding obtained for each tRNA-synthetase complex was compared with the results of direct ultraviolet cross-linking experiments with these complexes.

The interaction between aminoacyl-tRNA synthetases and their cognate tRNAs has been the subject of considerable investigation over the past years [for recent general reviews, see Kisselev & Favorova (1974), Schimmel (1977), Ofengand (1977), and Goddard (1975)]. Among the approaches investigated, the use of partial digestion with nucleases as a method for the study of complex formation was applied to several systems by Hörz & Zachau (1973), Dube (1973), and Dickson & Schimmel (1975). The principle of this approach is that regions of the tRNA which are cut by the RNase become resistant when they are shielded by the protein. In all the systems studied so far, the ribonulceases employed (A, T_1, or T_2) generate cleavages in mild digestion conditions only in the nonhelical sections of the tRNA (Hörz & Zachau, 1973; Dickson & Schimmel, 1975).

The isolation from cobra Naja oxiana venom by one of the authors (S.V.) of an RNase without base specificity which preferentially cuts structured regions of an RNA (Vassilenko & Babkina, 1965; Vassilenko & Ryte, 1975) made it possible to determine the parts of the tRNA involved in the cloverleaf helical sections. We have digested yeast $tRNA^{Phe}$ and $tRNA^{Val}$, with these nucleases both in the free state and complexed with their synthetases. We compared these results

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with those from photochemical cross-linking studies on the same systems.

Materials and Methods

Yeast $tRNA_1^{Val}$ and $tRNA^{Phe}$ were purified from total brewer's yeast tRNA (Boehringer Mannheim Corp.) by countercurrent distribution followed by conventional chromatographic techniques.

Valyl- and phenylalanyl-tRNA synthetases from yeast were isolated according to Kern et al. (1977). The turnover numbers of purified enzymes measured at 20 °C and pH 8 were usually around 5 s^{-1}.

tRNAs were labeled at the 5′ end with ^{32}P by standard techniques (Silberklang et al., 1977) and at the 3′ end by using tRNA-nucleotidyl transferase.

Partial Cobra Venom and T_1 RNase Digestions. Prior to use, ^{32}P-labeled tRNAs were heated at 55 °C for 2 min in 10 mM potassium phosphate buffer, pH 7.0, then 10 mM $MgCl_2$ was added, and the solution was slowly cooled. Labeled tRNA (7 μM) was incubated in the same buffer in the presence or absence of the appropriate tRNA synthetase. Synthetases were generally present in 2-fold excess over tRNA concentration. Nucleases were added at time zero as specified in the legend of the figures. Digestions were stopped by adding an equal volume of water-saturated phenol to the aliquots (50 μL). Samples were shaken at room temperature for 2 min and then centrifuged, and ethanol was added to the aqueous phase and precipitated at −70 °C. The precipitates were collected by centrifugation and dried in vacuo.

The ^{32}P-labeled fragments were separated on 15% or 20% polyacrylamide slab gels as described above. For identification of the cuts, a "ladder" showing every possible intermediate (obtained by limited alkaline hydrolysis in 50 mM $NaHCO_3/Na_2CO_3$, pH 9.0, 1 mM EDTA, or in boiled distilled water) and partial digests with T_1 RNase (in 20 mM sodium citrate, pH 5.0, 7 M urea, and 1 mM EDTA for 15 min at 50 °C; Donis-Keller et al., 1977) were also separated on the same gel. T_1 nuclease digestion fragments and alkaline digestion fragments have a 3′-phosphate whereas cobra venom RNase fragments terminate with a 3′-hydroxyl group. This difference in the RNase's digestion products raises problems especially for small oligonucleotides: the small cobra venom RNase fragments often migrate between two adjacent bands of alkaline hydrolysis fragments. However, oligonucleotides longer than 8–10 nucleotides migrate the same regardless of their origin. An example was the 5′-end-labeled tRNA (Figure 2A). Furthermore, in the case of 3′-labeled tRNAs ($tRNA^{Val}$ and $tRNA^{Phe}$), the ladders did not show the last five oligonucleotides near the 3′ end and this seems to be a general property of all tRNAs since $tRNA^{Asp}$ from yeast also shows an incomplete alkaline ladder (S. Butorin, personal communication). Since oligonucleotides rich in C have rather small net charges in the absence of terminal phosphate groups, these oligonucleotides are retarded and stack together on the gel. The cobra venom RNase fragments of the same size were therefore analyzed by the wandering spot technique (Silberklang et al., 1977). How then was the alkaline scale complete when a 5′-end-labeled tRNA was used? The first possible intermediate is pXp for 5′-end-labeled tRNA while for a 3′-end labeled tRNA it is CpA. The charge:nucleotide ratio varies in the two cases from 4 to 1, which may explain the differences in the electrophoretic mobilities observed. It was expected for longer oligonucleotides that migration reflects the number of nucleotides owing to the high phosphate content. The experimental results gave support to our assumption: the T_1 fragment (position 68) in $tRNA^{Val}$ bearing 9 bases and 8 negative charges was only slightly shifted compared to a cobra venom RNase fragment of 10 bases and 11 negative charges (position 67) whereas in $tRNA^{Phe}$ the T_1 fragment corresponding to position 71 (5 bases and 4 negative charges) migrated to the same position as the cobra venom RNase fragment containing 8 bases and 9 negative charges (position 68) (see Results).

Results

For identification of the sites of the RNase attack on free tRNAs or tRNA–enzyme complexes, 5′- or 3′-end ^{32}P-labeled tRNAs were digested. The radioactive fragments were analyzed by the gel sequencing technique according to Donis-Keller et al. (1977) but by using much thiner gels (0.5 mm) and higher voltage (up to 1500 V).

This technique is simple and rapid and has the advantage of allowing identification of the primary cuts within the tRNA molecule. Secondary cuts will also be visualized if the resulting fragments bear the terminal label, but they are not visible in the complementary experiments where the label is on the opposite side of the RNA. This allows a discrimination between primary and secondary cuts: only cuts located at the same position using either 5′- or 3′-end labeled tRNAs can be considered as primary cuts.

Since preliminary results showed that an equimolar amount of enzyme afforded nearly the same tRNA protection against nuclease attack at pH 5.5 and 7.0, we preferred incubation at pH 7.0 which is closer to physiological conditions. In order to achieve maximal protection, the enzyme was used in excess.

The digestion patterns are shown in Figure 1A,B,C for the phenylalanine system and in Figure 2A,B for the valine system. The location of the cuts in the cloverleaf representation is shown in Figure 3A,B.

Partial Digestion of Yeast $tRNA^{Phe}$–Phenylalanyl-tRNA Synthetase Complex. 5′-[^{32}P]$tRNA^{Phe}$, in the absence of phenylalanyl-tRNA synthetase, is mainly cleaved in the anticodon stem (positions 28–30 and 41) and within a region spanning nucleotides 65–72, corresponding to the 3′ side of the acceptor stem (Figure 1A). The fragments arising from cuts in this latter region are only poorly separated so that the cutting positions cannot be located with accuracy. The use of 3′-^{32}P-labeled $tRNA^{Phe}$ allowed us to define with precision the nicks produced in this region and to obtain additional information, for example, to establish that there were several adjacent cuts (Figure 1C). The major cuts are found in positions 28 and 41 and in all positions from 66 to 71. From comparison of Figures 1A and 1C, it follows that the cuts in the anticodon and acceptor stems are primary ones. No definite conclusion can be drawn about cutting at residue G_{15} since the control also exhibits a band which corresponds in size to fragment 15–76 (not shown here).

Yeast $tRNA^{Phe}$ complexed with its cognate synthetase is fully protected against RNase action at the cleavage site 41 located at the 3′ side of the anticodon stem. A quantitative evaluation of the radioactivity corresponding to band 41 confirms a 100 protection at this site (see Table I).

The protection of $tRNA^{Phe}$ against RNase action by noncognate aminoacyl-tRNA synthetases was also studied. In the case of beef pancreas tryptophanyl-tRNA synthetase, no protection was observed (Figure 1A, slot B5).

The 5′-[^{32}P]$tRNA^{Phe}$ digest was also visualized by "Stains all" staining (Figure 1B). All the major fragments detected by autoradiography were seen. The other stained fragments observed in a 10-min digestion (slots A_1 and B_1) correspond to unlabeled fragments complementary to the labeled ones.

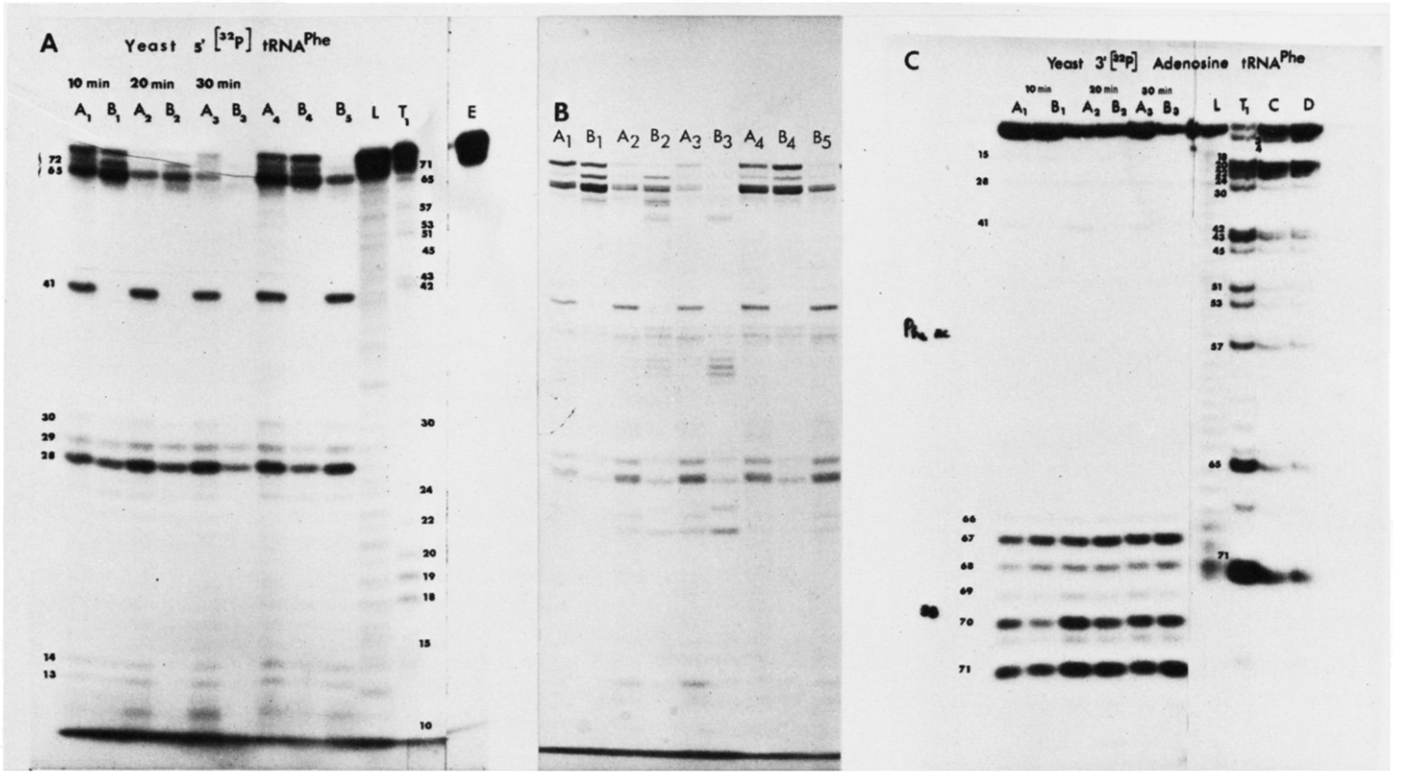

FIGURE 1: Partial cobra venom or T_1 RNase digestion of ^{32}P-labeled yeast $tRNA^{Phe}$ obtained in the absence or presence of yeast phenylalanyl-tRNA synthetase. (A) Autoradiograph of 5′-end labeled digestion products of $tRNA^{Phe}$ fractionated on a 15% polyacrylamide slab gel. $tRNA^{Phe}$ (475 pmol) was incubated with RNase at 0 °C without (A) or in the presence of 475 pmol of phenylalanyl-tRNA synthetase. (B) (A_1, B_1, A_2, B_2, A_3, B_3) incubation with 3 units of cobra venom RNase for 10, 20, and 30 min; (A_4, B_4) as for (A_2, B_2) but in the presence of 70 μM phenylalanine and 5 mM ATP–Mg^{2+}; (B_5 as for B_2 but instead of phenylalanyl-tRNA synthetase, 850 pmol of tryptophanyl-tRNA synthetase was added. (L) Partial alkaline ladder obtained by incubation of 5′-[^{32}P]$tRNA^{Phe}$ in boiled water for 20 and 40 min; (T_1) partial digestion of 200 pmol. 5′-[^{32}P]$tRNA^{Phe}$ at every guanine obtained with 0.05 and 0.1 unit of T_1 RNase in 7 M urea, sodium citrate–acetate, pH 5, and 1 mM EDTA after 5 min of incubation of 50 °C; (E) No treatment. (B) Staining of the same gel by "Stains-all". (C) Autoradiograph of partial cobra venom RNase digestion products of 3′-[^{32}P]adenosine labeled $tRNA^{Phe}$. The establishment of the cleavage sites 66–71 by the cobra venom RNase was done by the "wandering spot analysis." (A, B, C, L, T_1, E) As for the 5′-labeled $tRNA^{Phe}$, 1 unit of ribonuclease was used in this experiment. Slots C and D correspond to the incubation of 300 pmol of tRNA with 2.5 units of T_1 RNase for 5 min at 0 °C in the absence and presence of the synthetase.

Table I: Radioactivity Measurements of the Digestion Products Corresponding to Figure 1C[a]

	cobra venom RNase (cpm)		T_1 RNase (cpm)	
fragment	−syn-thetase	+syn-thetase	−syn-thetase	+syn-thetase
76–18				
76–19			91 880	72 600
76–20				
76–28	2 070	2 350		
76–41	2 780	0		
76–67	33 850	39 000		
76–70	37 800	33 800		
76–71	73 400	71 600		
residual $tRNA^{Phe}$	127 000	154 000	10 413	126 700

[a] The counts per minute (cpm) listed correspond to a 10-min digest in the case of cobra venom RNase. Background has been subtracted. No difference was observed with a longer digestion time.

Prolonged incubation gives additional fragments due to secondary nicks.

The effect of aminoacylation of tRNA on the topography of the Phe-$tRNA^{Phe}$–synthetase complex was also studied. The $tRNA^{Phe}$ was aminoacylated in situ before addition of the nuclease. Slots A_4 and B_4 show the fragments produced after 20-min digestion of the aminoacylated tRNA in the absence and the presence of the synthetase (Figure 1A). No new cuts appear compared to the uncharged form. Here again, position 41 is strongly protected in the charged tRNA.

The action of T_1 RNase was investigated in order to get additional information concerning specific sites in the tRNA molecule, especially in the loops, but also to compare our results with those obtained in the $tRNA^{Phe}$ system by Hörz & Zachau (1973) using a nonradioactive method. These authors reported that cleavage occurred in free tRNA mainly at positions 18–21 in the dihydrouridine loop and also to a minor extent at position 57 in the TΨ loop. Of these two sites, only the D loop was shielded by the protein. We confirmed that in free tRNA the main splitting occurs at positions 18–21 in the D loop (Figure 1C, slot C), but we could not conclude simply by gel inspection that there was protection by the phenylalanyl-tRNA synthetase at these sites. Therefore, radioactive measurements of the same fragments were made (see Table I). In the presence of the synthetase, a 20% difference was observed. This difference is in the range of the counting error (15%) so that no definite conclusion can be reached concerning protection. Hörz & Zachau (1973) reported a protection of 70%. Perhaps the kinetics of digestion should also be followed before drawing any conclusion.

Partial Digestions of $tRNA^{Val}$–Valyl-tRNA Synthetase Complex. In the absence of valyl-tRNA synthetase, 5′-[^{32}P]$tRNA^{Val}$ was cleaved mainly in the region located between position 64 and a position near the 3′ end which could not be characterized with precision and to a lesser extent at positions 28–30 and 41–42 (Figure 2A). Minor cuts were found at

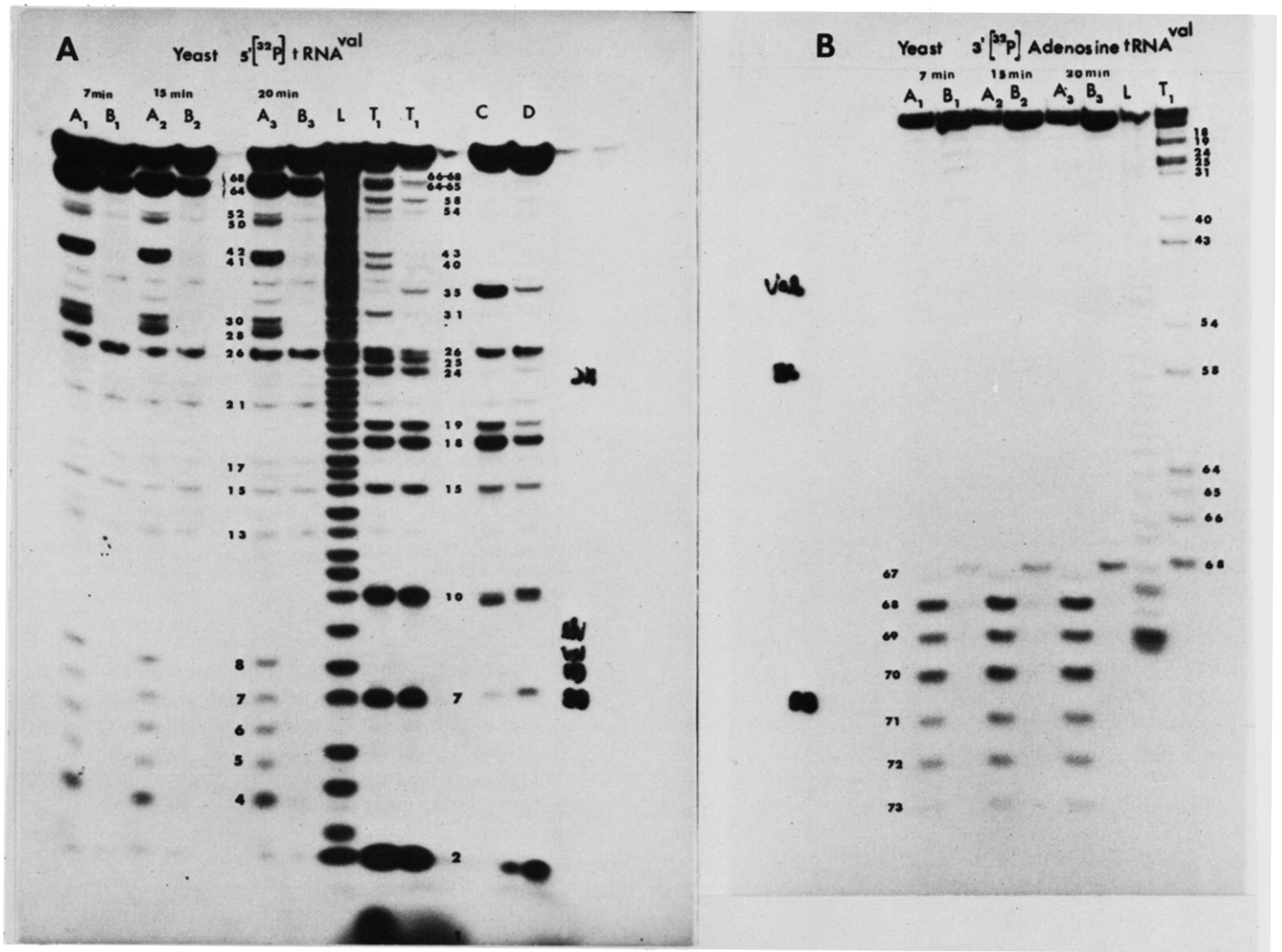

FIGURE 2: Partial cobra venom digestion of ^{32}P-labeled yeast tRNA^{Val} obtained in the absence or presence of yeast valyl-tRNA synthetase. (A) Autoradiograph of 5′-labeled digestion products of tRNA^{Val} fractionated on a 15% polyacrylamide slab gel. tRNA^{Val} was incubated with RNase at 0 °C without (A) or in the presence of 600 pmol valyl-tRNA synthetase. (B) (A_1 B_1 A_2 B_2 A_3 B_3) Incubation with 2.8 units of cobra venom RNase for 7, 15, and 20 min. (L, T_1) As in Figure 1A. (C, D) Correspond to the incubation of 300 pmol of tRNA with 1.5 units of T_1 RNase for 5 min at 0 °C in the absence and in the presence of the synthetase. The cut at position 26 is not due to cobra venom or T_1 hydrolysis as it is present in the control (not shown). (B) Autoradiograph of 3′ adenosine-labeled tRNA^{Val} digestion products fractionated on polyacrylamide slab gel. The same legend as in Figure 2B applies here. The cleavage sites 67–73 were established by the "wandering spot analysis".

positions 4–8 corresponding to the 5′ side of the acceptor stem and at positions 50–52 corresponding to the 5′ side of the TΨ stem. The weakness of the cleavage sites at C_{13}, G_{15}, and D_{21} in the D loop does not allow us to consider them as a result of the cobra venom nuclease activity, as one or more contaminating nucleases may be present in our enzyme preparations. Examination of the digestion pattern of 3′-[^{32}P]tRNA^{Val} allowed the identification of the cuts near the 3′ end. These cuts are located between positions 67 and 73. The other cleavage points observed with the 5′-labeled tRNA are very weak and cannot be seen in Figure 2B. This suggests that the cuts observed in the region 67–73 are primary cleavage positions, the other being secondary ones and resulting from cleavages of the large 1–67 to 1–73 fragments.

The addition of valyl-tRNA synthetase leads to complete protection of positions 28–30 and 41–42 both located in the anticodon stem, positions 50–52 located in the 5′ side of the TΨ stem, and positions 4–8 located in the 5′ part of the acceptor stem, as shown in the digestion pattern of 5′-[^{32}P]-tRNA^{Val}. Examination of the 3′-[^{32}P]tRNA^{Val} digests shows that the region at the 3′ end of tRNA^{Val} from 68 to 73, corresponding to the 3′ side of the amino acid acceptor stem, was also protected. On the other hand, splitting at position 67 is speeded up in the presence of the synthetase. This could be the result of either a local conformational change in the tRNA or competition between several sites within the tRNA for the ribonuclease. Shielding of high affinity sites (positions 68–73) by the synthetase could lead to enhancement of hydrolysis at other sites having a lesser affinity, such as those located at position 67.

T_1 RNase splits free tRNA^{Val} (Figure 2A, lane C) in the anticodon loop at the I_{35} site, as well as in the D loop (positions 15, 18, and 19). The weak cleavage sites observed at residues G_2G_7 and G_{10} correspond to helical regions. In the presence of the synthetase, positions 18, 19, and 35 seem to be protected. But the same reservations are to be made as for T_1 tRNA^{Phe} digestions.

Discussion

One can remark the unique properties of the cobra venom RNase compared to other RNases such as T_1 or pancreatic RNase. The kinetics of digestion of tRNA^{Val} and tRNA^{Phe} obtained with this nuclease show remarkably constant patterns of hydrolysis, probably as a consequence of its specificity for structured regions. A cut in an helical region of the tRNAs strongly diminishes the affinity of the enzyme for this region.

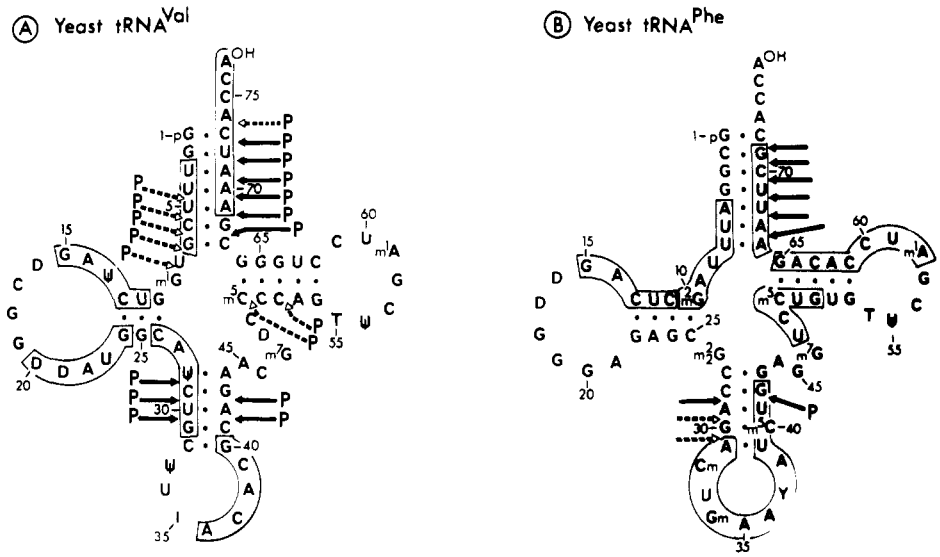

FIGURE 3: Cloverleaf structures of yeast $tRNA^{Phe}$ (A) and $tRNA^{Val}$ (B) according to RajBhandary et al. (1967) and Bonnet et al. (1974) with the sites of attack for cobra venom RNase. P corresponds to the sites protected by the synthetases. The oligonucleotides cross-linked to the synthetase are indicated by the dashed areas. Minor cuts are represented by dotted arrows.

This is a very favorable situation as compared to the T_1 or pancreatic RNases which preferentially recognize single-stranded regions. To obtain well-characterized fragments with these nucleases it is necessary to adjust the enzyme concentration and digestion time in order to avoid nicking within the helical regions.

Common features are found for free $tRNA^{Phe}$ and $tRNA^{Val}$ with regard to their accessibility to the cobra venom enzyme (see Figure 3). Among the four stems present in these structures only three are cut, the amino acid acceptor, the anticodon, and the TΨ stems. These results confirm the specificity of the cobra venom enzyme for double-stranded regions. The 3′ side of the amino acid acceptor stem is the region most susceptible to cobra venom RNase whereas both sides of the anticodon stem are less attacked. The ratio of splitting deduced from Table I is 19, in favor of the former. The cuts in the TΨ stem of $tRNA^{Val}$ are very weak and represent less than 5%.

The D stem of both tRNAs is completely insensitive to RNase. As examination of the cutting positions confirms that the enzyme has no apparent sequence or base specificity, the different sensitivity of the four stems to cobra venom RNase hydrolysis can only be ascribed to features related to the tertiary structure of the tRNAs. The more distal parts of the L-shaped tRNA molecule (anticodon and amino acid acceptor steme are more sensitive than the TΨ and the D stems which are in close contact with each other.

We studied the protection afforded by the cognate aminoacyl-tRNA synthetase to the double-stranded regions of the tRNA against the cobra venom RNase. The protective effect in these experiments is very strong and reaches 100%; only the sites protected at such a level were considered as shielded regions. These regions are the acceptor, the anticodon, and the TΨ stems in $tRNA^{Val}$ whereas protection is limited to the anticodon stem in $tRNA^{Phe}$. Concerning protection within the anticodon stem, a difference is found between $tRNA^{Val}$ and $tRNA^{Phe}$: both sides are protected in $tRNA^{Val}$ protection is limited to the 5′ side. This suggests that the anticodon stem of $tRNA^{Val}$ is less exposed during the interaction with the synthetase.

The amino acid acceptor stem is protected only in $tRNA^{Val}$. The lack of protection of this region in $tRNA^{Phe}$ is rather surprising, as an interaction between the 3′ end of tRNA and the enzyme must obviously take place in the course of the aminoacylation reaction. One has to keep in mind, however, that protection of tRNA by synthetase against ribonuclease is the result of a competition between the two enzymes for the tRNA. The protection depends on four parameters: synthetase and nuclease concentrations and K_{tRNA} for synthetase and for nuclease. Apparently the large excess of synthetase concentration (400 times over the K_{tRNA}) is not sufficient to prevent tRNA–nuclease interaction in the phenylalanine system. Therefore the tRNA–synthetase interaction in this system is rather weak compared to that in the valine system. This conclusion is strengthened by the comparison of the dissociation constants for the two systems. Under the experimental conditions used, K_D for $tRNA^{Phe}$ is 0.03 μM (Fasiolo et al., 1974) whereas K_D for $tRNA^{Val}$ is ≤0.001 μM (Bonnet et al., 1975).

If one represents the shielded regions in the three-dimensional model of tRNA (Figure 4), and this is particularly useful in the case of $tRNA^{Val}$ where the picture of protection is more complete than for $tRNA^{Phe}$, it appears that they are located on the insides of the two branches forming the L-shaped tRNA structure which agrees with the model of interaction proposed by Rich & Schimmel (1977).

The pattern of protection observed in both systems was interpreted as a consequence of interacting zones between tRNAs and their synthetases. It could be argued that the pattern of protection of $tRNA^{Phe}$ and $tRNA^{Val}$ in the presence of their synthetases results from a synthetase-induced conformational change of the tRNA. It is well-known that the anticodon loop in $tRNA^{Phe}$ undergoes some changes upon interaction with the synthetase (Krauss et al., 1977; Ehrlich et al., 1980), and recently a report has appeared showing that S_1 nuclease could be used as a probe for conformational differences in the anticodon of several tRNAs (Wrede et al., 1979). However, the fact that our results fit quite nicely with those obtained by direct tRNA–synthetase cross-linking experiments in the valine (Renaud et al., 1976; Ebel et al., 1979) and phenylalanine systems (Schoemaker et al., 1975; Ebel et al., 1979) gives support to our interpretation.

Tryptophanyl-tRNA synthetase from beef pancreas was used to test the specificity of interaction between $tRNA^{Phe}$ and its cognate synthetase, keeping in mind that binding can occur with noncognate enzyme (see Ebel et al., 1973). Besides,

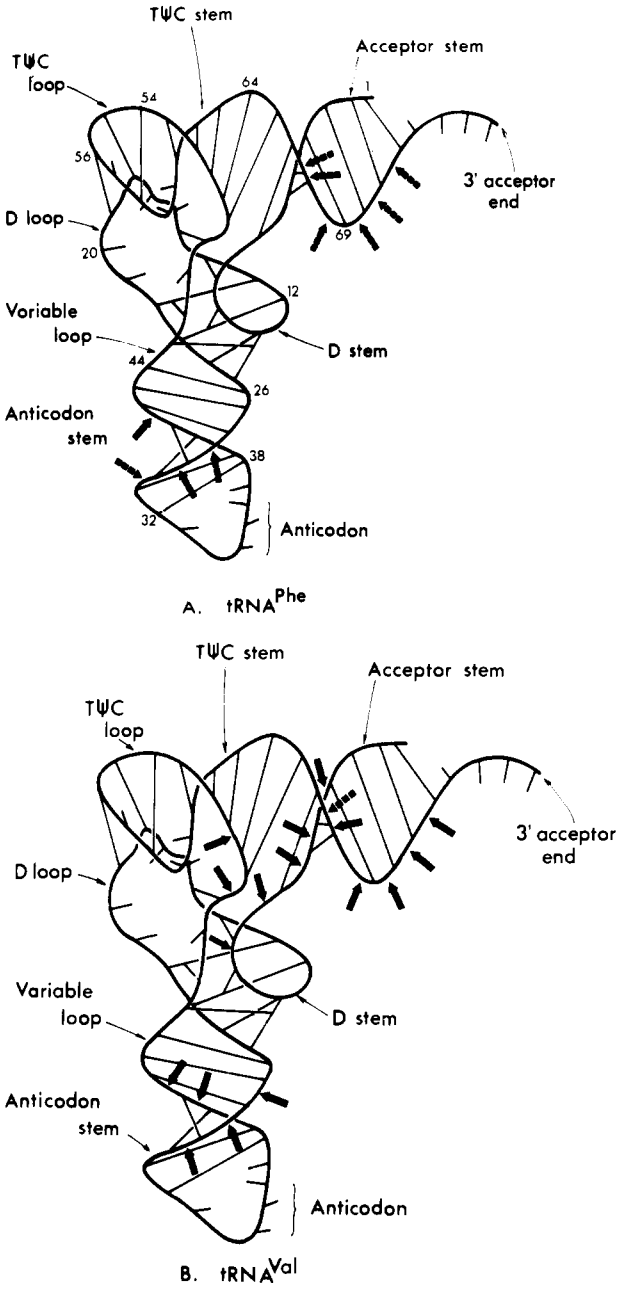

FIGURE 4: Localization on the three-dimensional structures of the tRNAs of the cleavage sites and of the sites protected by the cognate synthetase. (A) Three-dimensional model of 4 Å of $tRNA^{Phe}$ according to Kim et al. (1973). (B) It is believed that the three-dimensional model of $tRNA^{Phe}$ applies as a first approximation to the tertiary structure of $tRNA^{Val}$. The black arrows indicate the protected cleavage sites; the dotted arrows indicate those not protected by the synthetase.

$tRNA^{Phe}$ was misaminoacylated with the beef enzyme. Indeed, tryptophanyl-tRNA synthetase charges $tRNA^{Phe}$ with the following kinetic parameters: K_m for tRNA of 20 μM and a V_{max} which is reduced by a factor of 5000 compared to the homologous tRNA. By using the K_m values as an approximation of the K_D for $tRNA^{Phe}$, one calculates that nearly 50% of the tRNA molecules are bound to the protein.

This level of saturation is apparently not sufficient for protection since the tRNA complex is in dynamic equilibrium with the dissociated species, and this equilibrium is shifted toward the unbound form as the free tRNA is digested.

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